Biomedicine & Pharmacotherapy 105 (2018) 115–120

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Glucose-regulated protein of 94 kDa contributes to the development of an aggressive phenotype in breast cancer cells

Pedro Buc Calderon[a,b,*], Anne-Laure Sennesael[b], Christophe Glorieux[b,1]

[a] Facultad de Ciencias de la Salud, Universidad Arturo Prat, 1100000, Iquique, Chile
[b] Metabolism and Nutrition Research Group, Louvain Drug Research Institute, Université catholique de Louvain, 1200, Brussels, Belgium

ARTICLE INFO

Chemical compounds studied in this article:
Cobalt chloride (PubChem CID: 24288)
Doxorubicin (PubChem CID: 443939)
Staurosporine (PubChem CID: 44259)

Keywords:
Grp94
Cancer cells
Hypoxia
Glucose
Aggressive phenotype
Invasion
Doxorubicin

ABSTRACT

Grp94 plays an essential role in protein assembly. We previously suggested that Grp94 overexpression is involved in tumor aggressiveness. However, the underlying mechanisms remain unknown. Since many tumors display high Grp94 levels, we investigated the effects of tumor microenvironment on the regulation of this chaperone expression. First, we found out that hypoxia did not change Grp94 expression in the human tumor cell lines MCF-7 (breast cancer) and HepG2 (liver cancer). Second, glucose deprivation significantly increased Grp94 protein levels. Subsequently, we focused in the putative role of Grp94 in the acquisition of an aggressive phenotype by cancer cells. Using a more aggressive cancer cell model (MDA-MB-231 breast tumor cells), we found out that Grp94 knockdown using siRNA decreased the invasive capacity of cancer cells. Moreover, cells with decreased Grp94 levels displayed an enhanced sensitivity of tumor cells to doxorubicin, a standard drug in the treatment of breast cancer. Taken together, our results suggest that the expression of Grp94 is linked to tumor aggressiveness. Therefore, targeting Grp94 could be an effective way to inhibit tumor growth improving chemotherapy outcome.

1. Introduction

Grp94 (glucose-regulated protein of 94 kDa) participates in the detection of misfolded proteins in the endoplasmic reticulum (ER), sending them to the proteasome for their degradation [1,2]. This chaperone protein was initially described in fibroblasts transformed with Rous sarcoma virus, and its overexpression was associated with glucose depletion in the medium [3]. Hence, this chaperone is referred as a "glucose-regulated" protein.

Grp94 may have functions that are different from those of chaperones related to heat shock proteins such as Hsp90, Hsp70 or Grp78 [4]. Indeed, the levels of Grp94 are higher in tumor cells [5–9] and correlate with the advanced stage and poor survival of cancer patients [5,10,11]. In this regard, *in vitro* studies have shown that Grp94 expression is 3 to 5 times higher in breast cancer cells (MCF-7, MDA-MB-231) compared to the normal breast cell lines HBL-100 or human mammary epithelial cells [12]. However, the molecular mechanisms regulating such Grp94 overexpression in cancer cells are still not fully elucidated.

Grp94 also provides cancer cells resistance to radiation therapy and chemotherapy [13,14]. In addition, Grp94 is overexpressed in metastasis and relapses compared to primary tumors [5,15]. Considering the clinical therapeutic context, the inhibition of Grp94 may have some advantages as a pharmacological target for treating cancer. For instance, Grp94 does not appear to be necessary for the survival of healthy cells [16]. Moreover, cells lacking Grp94 expression do not have major problems in protein folding and ER stress is not induced [17]. However, in mice lacking Grp94 expression, such inhibition can cause some undesirable effects such as thrombocytopenia [18]. Therefore, a better understanding of the role played by Grp94 in tumor progression, as well as its regulation, is required prior considering this chaperone as a new therapeutic target in anticancer therapy.

The purpose of this study was twofold. First, we investigated the regulation of Grp94 in tumors, and more specifically the role of tumor microenvironment in the increased levels of Grp94. Two different human cell lines were selected: MCF-7 breast cancer and HepG2 hepatoma cells. Second, we focused on the putative role of Grp94 in the acquisition of an aggressive phenotype by tumor cells. To this end, we assessed the influence of Grp94 on invasion and resistance

Abbreviations: eIF2, eukaryotic initiation factor 2; ER, endoplasmic reticulum; Grp94, glucose-regulated protein of 94 kDa; Grp78, glucose-regulated protein of 78 kDa; HIF-1α, hypoxia inducible factor-1 alpha; HRE, hypoxia response element; Hsp, heat shock protein; UPR, unfolded protein response

* Corresponding author at: MNUT B1.73.11, Avenue E. Mounier, 73, 1200, Brussels, Belgium.
E-mail address: pedro.buccalderon@uclouvain.be (P. Buc Calderon).
[1] Present address: Sun Yat-Sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center of Cancer Medicine, 510275 Guangzhou, China.

https://doi.org/10.1016/j.biopha.2018.05.106
Received 8 March 2018; Received in revised form 18 May 2018; Accepted 18 May 2018

to doxorubicin using the highly metastatic MDA-MB-231 breast cancer cells.

2. Materials and methods

2.1. Cell culture

Human derived cell lines DU-145 (gallbladder), HepG2 (liver), Ishikawa (endometrial), T24 (prostate), K562 (chronic myeloid leukemia), MCF-7 and MDA-MB-231 (breast) were purchased from ATTC (Manassas, VA, USA). Cells were cultured as monolayer and kept in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal calf serum, in the presence of penicillin (100 U/ml) and streptomycin (100 µg/ml) from Gibco (Grand Island, NY, USA). K562 cells were incubated as cells suspension in RPMI milieu (Gibco). All cultures were maintained at 37 °C in 95% air/ 5% CO_2 with 100% humidity.

For the evaluation of the effect of hypoxia on Grp94 expression, cells were incubated in a hypoxic chamber INVIVO2 500 (Ruskinn), under 1% O_2 and 5% CO_2. Cobalt chloride ($CoCl_2$), staurosporine and doxorubicin were from Sigma (St Louis, MO, USA).

2.2. Western blot

Both MCF-7 and HepG2 cells were incubated for three different periods of times (6, 24 and 72 h) under four different experimental conditions: control (no addition), hypoxia (1% O_2), $CoCl_2$ (150 µM) and in glucose-free medium. The procedures for protein sample preparation from cell cultures, protein quantification, immunoblotting and data analyses were performed as reported elsewhere [21,22]. Antibodies against Grp94 and Grp78 were obtained from Cell Signaling Technology (Danvers, MA, USA); β-actin antibody was from Abcam (Cambridge, UK); and HIF1α antibody was from BD Transduction Laboratories (Lexington, USA). Protein bands were then detected by chemiluminescence using the ECL detection kit (Pierce, Thermo Scientific, Rockford, IL, USA). When appropriate, bands were quantified, using ImageJ software (http://rsb.info.nih.gov/ij/). Protein expression was normalized to that of β-actin.

2.3. siRNA assay

Dharmafect reagent 1 was used for transfection of siRNA against Grp94 (ON-TARGET plus SMART pool siRNA) in MDA-MB-231 cell line, according to the protocols provided by Dharmacon (Lafayette, CO, USA). Briefly, 3.5×10^5 cells were incubated for 24 h up to 50% confluence and transfection was performed with a 0.1 µM siRNA solution. All experiments were performed 48 h after transfection.

2.4. MTT reduction assay

The effect of doxorubicin on viability of MDA-MB-231 cells was assessed by following the reduction of MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) to blue formazan [19]. Cells were first transfected either with a siRNA control or directed against Grp94 (as reported in point 2.3), and after 48 h of transfection, the culture medium was replaced by a fresh medium containing doxorubicin (1, 3, and 10 µM) and further incubated for 24 h. Thereafter, 100 µl of MTT solution (0.5 mg/ml) was added into the wells and cells were further incubated for 2 h at 37 °C. Wells were rinsed with PBS and the blue formazan crystals were solubilized with 100 µl of dimethyl sulfoxide (DMSO). The colored solution was subsequently read at 550 nm. Results were expressed as % of MTT reduction compared to untreated control conditions.

2.5. Caspase-3 assay

DEVDase activity, which reflects the caspase-3 activity, was monitored after 16 h incubation of MDA-MB-231 transfected cells with either doxorubicin (1 and 10 µM) or staurosporine (1 µM). Cells were then washed twice with PBS, lysed and centrifuged. Subsequently, cell supernatants were incubated with Asp-Glu-Val-Asp-AFC (DEVD-AFC), and substrate cleavage was determined kinetically at room temperature using Victor X2 spectrophotometer (380 nm excitation, 500 nm emission) (Perkin Elmer, Waltham, MA, USA). Results are expressed in U/mg proteins as originally described by Nicholson et al [20]. The amount of proteins was determined by using the Bio-Rad Protein Assay.

2.6. Invasive capacity assay

To evaluate the relation between Grp94 and cancer cells invasiveness, experiments were performed using the Boyden chamber allowing to monitor the tumor cells passage across an *in vitro* matrix. Briefly, matrigel invasion assays were performed using modified Boyden chambers with polycarbonate nucleopore membranes (Corning, USA). First, pre-coated filters were rehydrated with 500 µl medium. MDA-MB-231 transfected cells (5×10^4) in 500 µl medium were seeded into the upper part of the chamber, and the lower compartments were filled with 500 µl medium supplemented with 10% FBS. Following 24 h incubation at 37 °C, non-invaded cells on the upper surface of the filter were wiped off with a cotton swab, and the invaded cells on the lower surface of the filter were fixed and stained with crystal violet. Invasiveness was determined by counting cells in five microscopic fields per well, and the extent of invasion was expressed as an average number of cells per microscopic field.

2.7. Statistics

All experiments were performed at least in triplicates. Groups were analyzed using unpaired *t*-test performed with GraphPad Prism software (San Diego, CA, USA). The level of significance was set at $p < 0.05$.

3. Results

3.1. The expression of Grp94 is regulated neither by hypoxia nor by activation of HIF-1α transcription factor

Fig. 1 (MCF-7 cells) and Fig. S1 (HepG2 cells) illustrate the expression levels of Grp94 protein in cancer cells incubated for different periods of times under either normal (CTL) or hypoxic (HYP) conditions. HIF-1α expression was used to control the experimental hypoxia (Figs. 1A and S1A). Our results show that Grp94 expression remained unchanged irrespective of the pO_2 levels, the duration of the incubation times and the origin of the human tumor cell lines (Figs. 1B–C, S1B–C).

To gain further insights into the roles of hypoxia and HIF-1α as potential regulators of tumor Grp94 overexpression, another HIF-1α modulating condition, namely a chemical-like hypoxia model by using cobalt chloride ($CoCl_2$) was explored. In line with the above experiments, Figs. 1 and S1 show that Grp94 expression remains unchanged in cells incubated under normal and hypoxic conditions. Specifically, Grp94 levels are similar in both MCF-7 (Fig. 1D–F) and HepG2 (Fig. S1D–F) cells incubated for 6 h or 24 h. This result is observed either in the absence (untreated cells) or in the presence of $CoCl_2$ (150 µM).

3.2. The expression of Grp94 is controlled by glucose depletion

Grp94 belongs to "glucose-related" proteins. Thus we explored the effect of glucose depletion on these protein chaperones expression. To this end, MCF-7 and HepG2 cells were incubated in a glucose-free DMEM medium.